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Pancreatic *reg* Gene Expression Is Inhibited During Cellular Differentiation

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Background and Objective

Factors that control pancreatic regenerating (*reg I*) gene expression are unknown, but it is believed that its expression may correspond with cellular differentiation. The authors recently demonstrated that *reg I* is expressed in AR42J, a rat acinar cell line whose state of differentiation can be modulated by dexamethasone. They used this line to study *reg I* expression during cellular proliferation and differentiation.

Methods

After treatment of cells with 10 nmol/L dexamethasone, proliferation was assayed by thymidine incorporation; differentiation by expression of elastase I mRNA. *Reg I* mRNA levels were measured using a rat *reg I* cDNA probe, and *reg I* protein levels assayed by enzyme-linked immunosorbent assay of cellular lysates with a polyclonal antibody. The effect of gastrin, cholecystokinin and glucagon on *reg I* expression was also studied.

Results

When compared with controls, treatment with dexamethasone caused thymidine incorporation to decrease and elastase mRNA levels to increase. *Reg I* mRNA decreased from controls of 100 ± 16% to 40 ± 18% ($p < 0.05$), and *reg I* protein levels decreased as well. Gastrointestinal hormones had no significant effect on either elastase or *reg I* gene expression.

Conclusions

Expression of *reg I* inversely correlates with the level of cellular differentiation, can be modulated via the glucocorticoid receptor, and is a potential marker of gastrointestinal epithelial differentiation. Despite its presence within a pancreatic acinar cell line, *reg I* gene expression is not modulated by gastrointestinal hormones.

The pancreatic regenerating (*reg I*) gene and its protein product are expressed within the exocrine cells of the pancreas.[1,2] *Reg I* is part of a family of at least three proteins described in human, rat and mouse, that bear sequence homology to calcium-dependent lectins.[2] Whereas the mRNA sequence of *reg I* was originally described by Terazano et al.,[1] its amino acid sequence is identical to pancreatic stone protein and pancreatic thread protein. The sequence of *reg III* is identical with pancreatitis associated protein, and is approximately 60% homologous to *reg I.* To date, the *reg II* homologue has only been described in the mouse.[2]

Reg I mRNA is overexpressed within pancreatic cells following pancreatitis or resection, and its protein product is believed involved in the regeneration of islets from ductular precursor cells.[1,2] Factors that control production of this acinar cell product are, to date, unknown. Because it is found in regenerating pancreatic tissue, Rouquier et al.[3] proposed that rat *reg I* is expressed within pancreatic cells during the dedifferentiated state. Watanabe et al.[4] discovered ectopically expressed *reg I* mRNA in human colon and rectal tumors, which further suggested that *reg I* gene expression correlates with the state of cellular differentiation.

We studied the levels of intracellular *reg I* mRNA and protein during pancreatic acinar-cell differentiation. In a previous study, we showed that the rat pancreatic acinar cell line AR42J cells express *reg I* mRNA.[5] Logsdon, et al.[6–8] have shown that treatment of these cells with 10 nmol/L dexamethasone for at least 18 hours causes them to differentiate, as evidenced by a decrease in thymidine incorporation and an increase in amylase expression.

In this study, we treated AR42J cells with dexamethasone to induce cellular differentiation, and then studied cellular changes in *reg I* gene expression. Because AR42J expresses receptors to gastrointestinal hormones, we also studied the effect of gastrin, cholecystokinin and glucagon on differentiation and *reg I* expression on these cells.

MATERIAL AND METHODS

Cell Culture

AR42J cells were purchased from American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM with 5% FCS, in triplicate for each experiment. Stock solutions of dexamethasone (Sigma, St. Louis MO) were constructed by dissolving in 50% ethanol and diluted at least 1000-fold for *in vitro* use. Controls were treated with an identical carrier solution. To determine the best inhibitory dose of dexamethasone, cells were initially cultured in 1-, 10- and 100-nmol/L concentrations for 24 hours In other experiments, cells were incubated in 10 nmol/L of the gastrointestinal hormones gastrin, cholecystokinin, or glucagon (Sigma, St. Louis, MO), in 1% bovine serum albumin, for 24 hours prior to harvest. In these experiments as well, controls were treated with an identical carrier solution.

Thymidine Incorporation

In 1 cc media, 5×10^5 AR42J cells were plated in 24-well plates and treated as detailed previously. [^{3}H]-Thymidine incorporation was assayed by scintillation after 24 hours incubation, as earlier described.[5]

RNA Analysis

Total RNA was isolated from 10^7 AR42J cells grown in 75-mL flasks by the TRI-REAGENT technique (Molecular Research Corp., Cincinnati, OH)[9]; integrity of RNA was confirmed by formaldehyde-agarose gel electrophoresis. *Reg I* mRNA expression was determined using a 262 base pair cDNA probe, constructed as previously described by reverse-transcriptase polymerase chain reaction[10] from rat pancreatic RNA using primers derived from the published sequence.[1] This sequence is homologous to mouse *reg I* published by Undo et al.,[2] and is in a region of the *reg* family unique to *reg I.* Specifically, there is little homology to *reg III*, or rat pancreatitis associated protein.[2,11] The primer sequences were: upstream: 5′-CTGGCCTCTCTGATTAAGGAG-3′, and downstream: 5′-CAGATGATTTCAGGCTTGAA-3′.[1] Elastase I was assayed in a similar fashion using a 573 bp cDNA probe, constructed by reverse-transcriptase polymerase chain reaction[10] from pancreatic RNA, using primers based on the published sequence (upstream 5′-GTGAGCAGCCAGATGACTTTC-3′, downstream 5′-CCTGGATGAACAATGTCATTG-3′).[12]

The polymerase chain reaction products were ultrafiltered using a 30,000 molecular weight filter (Millipore, Bedford, MA) to remove unincorporated dNTPs, and radiolabeled by random-priming (PRIME-IT, Stratagene, La Jolla CA) with δ-^{32}P-dCTP (Amersham, Arlington Heights, IL).

Quantitative analysis of gene expression was determined by slot blot analysis. Twenty micrograms (μg) of RNA were loaded per slot. Blots were prehybridized in buffer (5× SSPE, 30% formamide, 5× Denhardt's solution, 1% SDS and 100 μg/mL salmon sperm DNA) for

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2 hours at 37 C. Overnight hybridization was performed at 37 C with ^{32}P-radiolabeled probe (10^6 counts per minute/mL). After hybridization, the blots were washed twice in 2 × SSPE plus 0.2% SDS at 56 C for 20 minutes, and with 0.1 × SSPE at 56 C for 15 minutes, and then again for 10 minutes. Autoradiography was performed at −70 C with enhancing screens. Radioactivity was quantitated with a Betagen scanner (Betagen Corp., Waltham MA). To correct for loading, the blots were stripped, reexposed to ensure removal of signal, and reprobed with δ-^{32}P-radiolabeled oligo-dT, and recounted. Data were expressed as corrected counts (counts per minute reg/counts per minute oligo-dT), after subtracting background, and reported as mean ± SEM.

Protein Analysis

After treatment of 1×10^6 AR42J cells with 10 nmol/L dexamethasone for 5 days, total protein was isolated in harvested cells by lysis in 0.5 mL 1% NP-40 (Sigma). Ten micrograms of cellular lysate was electrophoresed on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose (Nytran, Schleicher and Schuell, Keene, NH). Western analysis was performed with a polyclonal antibody to human pancreatic stone protein (identical protein to *reg I*), a gift of J.C. Dagorn (Marseilles, France) using second antibody conjugated to alkaline phosphatase, and visualized (Pierce, Rockford, IL). Human pancreatic thread protein (PTP), an N-terminal truncated version of human *reg I*) was isolated as described previously,[5,13] and was used as a positive control. To compare *reg I* protein levels, an indirect enzyme-linked immunosorbent assay was constructed. Ten micrograms of lysate was plated and incubated at 4 C overnight, and washed with PBS. Antipancreatic stone protein (anti *reg I*) antibody in a 1:2000 dilution was then applied, and incubated for 1 hour. 1:4000 goat-anti rabbit antibody linked to alkaline phosphatase (Sigma) then added and used for visualization. Data were expressed as arbitrary optical density (OD) units (per 10 μg cellular lysate), after subtraction from background levels.

Statistics

Data are expressed as mean ± SEM. Comparison between groups was made using unpaired Student's t test, and significance defined as $p < 0.05$.

RESULTS

Morphology

When viewed by phase contrast microscopy, cultured AR42J cells appeared rounded in configuration, and grew as colonies in which the cells clumped together rather than in typical monolayers. After treatment with dexamethasone for 48 hours, microscopic changes were noted (Fig. 1). Cells divided at a slower rate, they became less clumped, flattened and developed intracellular vacuoles. This is consistent with findings of other investigators, who, using electron microscopy, showed increased endoplasmic reticulum and secretory granules after dexamethasone treatment.[6]

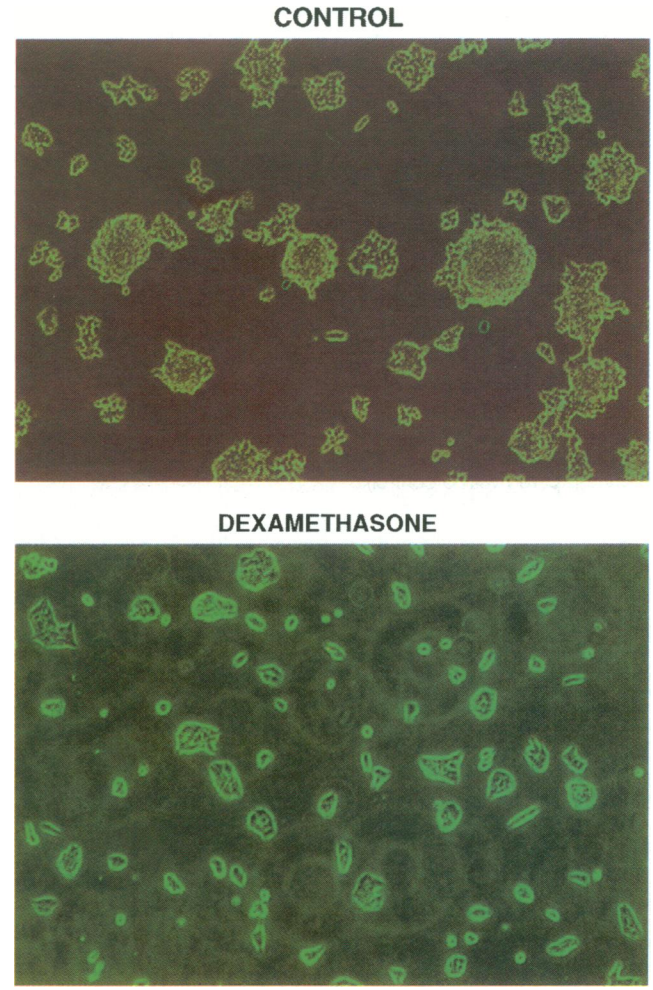

Figure 1. Photomicrograph (200×) of AR42J cells in culture before and after treatment with dexamethasone. After treatment, cells grow more slowly, become less clumped and flatten out.

Thymidine Incorporation and Gene Expression

Dexamethasone at 10 nmol/L significantly inhibited AR42J thymidine incorporation, as depicted in Figure 2. This is in agreement with the observations of others.[6–8]

Figure 3 shows a typical slot blot analysis of the effect of increasing doses of dexamethasone on *reg I* and elas-

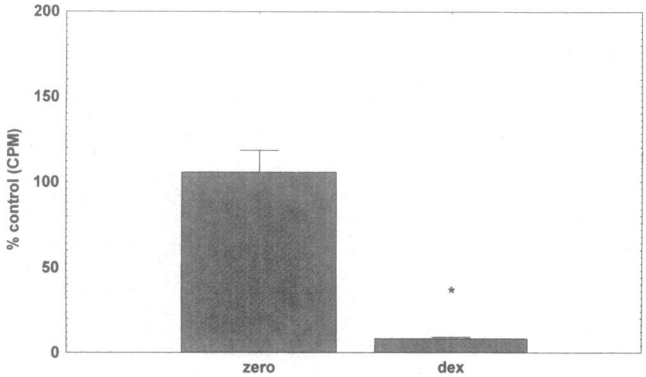

Figure 2. Effect of 10 nmol/L of dexamethasone on thymidine incorporation in AR42J cells. Percent change (mean ± standard error of the mean) is shown for 10 nmol/L dexamethasone treatment (*p < 0.05 compared with 0 control).

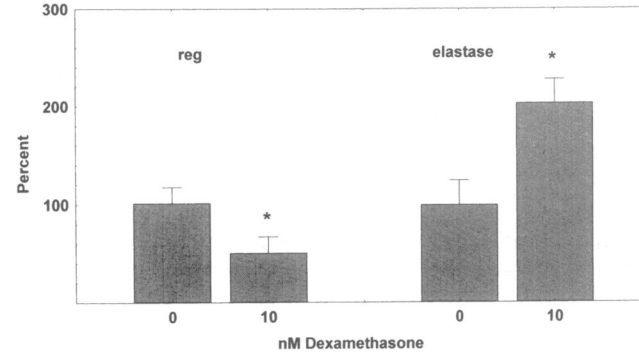

Figure 4. Percent change (mean ± standard error of the mean) in *reg I* and *elastase* gene expression, after dexamethasone treatment in five separate experiments (*p < 0.05 compared with 0 control).

tase I gene expression after 24 hours. At 10-nmol/L treatment and higher, a significant depression in *reg I* and a marked increase in expression of elastase I were noted. All further experiments were therefore performed at the 10-nmol/L dose. Figure 4 shows the mean ± SEM percent change of *reg I* and elastase mRNA levels in controls and 10 nmol/L dexamethasone for 5 separate experiments.

Reg I Protein Expression

Using a polyclonal antibody to human *reg I,* we confirmed its specificity to rat (pancreatic stone protein) first by immunofluorescent staining of frozen section of rat pancreas. This showed staining of acinar tissue, and not islet tissue (data not shown). Western analysis of AR42J lysate showed two bands at 14 to 17 kd (Fig. 5). Polyacrylamide gel electrophoresis and Western analysis of human pancreatic thread protein (an N-terminal truncated *reg I* protein)[2,13] showed a band at 13 kd (Fig. 5). Similar analysis of rat pancreatic juice, isolated as in the study performed by Bernard and colleagues of AR42J lysate showed a series of bands at 14 to 17 kd (not shown).[14]

Indirect enzyme-linked immunosorbent assay analysis revealed that after treatment with 10 nmol/L dexamethasone for 48 hours, mean ± SEM *reg I* protein decreased from a control OD of 263 ± 60 units/10 μg lysate to 83 ± 29 units/10 μg lysate ($p < 0.05$, $n = 5$ separate experiments).

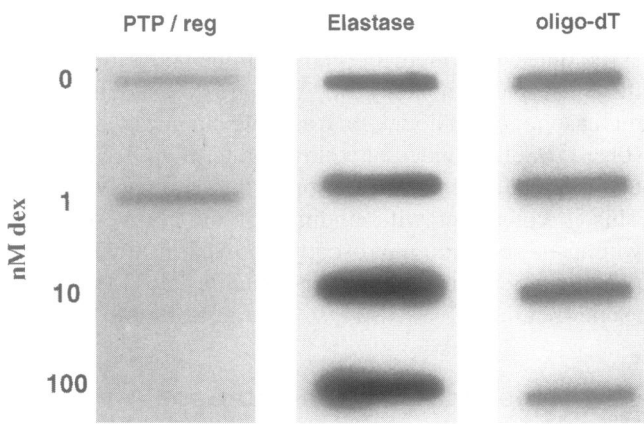

Figure 3. Effect of dexamethasone on *reg I* and elastase gene expression. Each row represents a single experiment. Twenty micrograms of RNA were loaded per slot, and probed with radiolabeled cDNA probe to *reg I,* stripped, reprobed with radiolabeled cDNA probe to elastase, stripped and reprobed with radiolabeled oligo-dT. Dexamethasone at 10 nmol/L effectively depressed *reg I* gene expression and induced *elastase I* gene expression (PTP/*reg* = *reg I*).

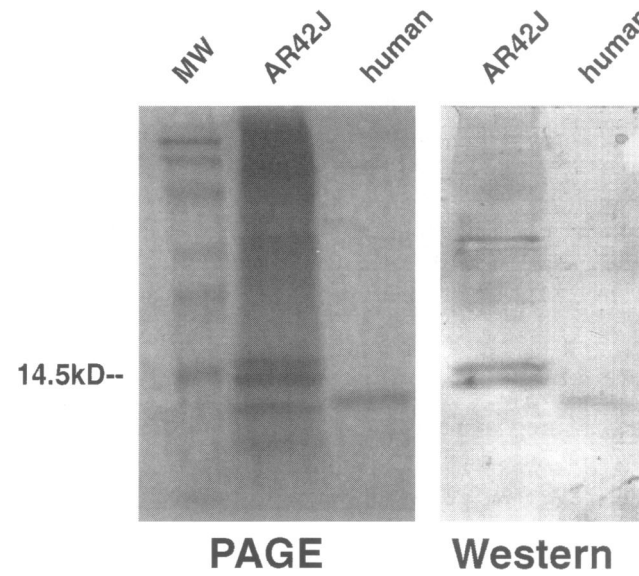

Figure 5. Western analysis of AR42J cell lysates for *reg I* protein using a polyclonal antihuman pancreatic stone protein antibody. For positive control, human pancreatic thread protein (an N-terminally truncated version of *reg I* ranging in size from 13 to 14 kD) was used. In AR42J, two bands were typically noted between 15 and 20 kD.

Table 1. EFFECT OF GASTROINTESTINAL HORMONES ON AR42J 10 NMOL/L GASTRIN, CHOLECYSTOKININ, AND GLUCAGON ON MEAN ± SEM [^{3}H]-THYMIDINE INCORPORATION, *REG I* AND ELASTASE I GENE EXPRESSION ON AR42J CELLS, AS DETERMINED BY SLOT BLOT

	Control	Gastrin	CCK	Glucagon
^{3}H incorporation	100 ± 11	150 ± 14*	106 ± 9	167 ± 13*
reg I	100 ± 40	63 ± 24	65 ± 31	66 ± 37
Elastase I	100 ± 17	64 ± 52	132 ± 10	77 ± 7

* Cells were incubated with 10 Nmol/L gastric cholecystokinin (CCK) or glucagon for 24 hr. Data expressed as mean ± SEM percent control.
* $p < 0.05$, compared to controls.

Effect of Gastrointestinal Hormones On AR42J Gene Expression

AR42J cells were treated with 10 nmol/L gastrin, cholecystokinin, and glucagon on AR42J for 24 hours. Table 1 shows the effects on of these gastrointestinal hormones on thymidine incorporation, on *reg I* gene expression, and on elastase gene expression in 5 separate experiments (mean ± SEM). Although gastrin and cholecystokinin increased thymidine incorporation, neither increased *reg I* gene expression.

Discussion

Reg I is normally expressed in pancreatic acinar tissue constituitively, but not in normal islets.[1] *Reg I* mRNA is overexpressed within pancreatic acinar cells and islets following pancreatitis or resection. Our laboratory recently showed that *reg I* mRNA is expressed in a rat pancreatic acinar cell line AR42J, but not the β-cell line RIN 1046–38 or the ductal cell line ARIP[5]. Using a polyclonal antibody to *reg I,* we presently show by Western analysis that AR42J cells also produce *reg I* protein.

We and others have recently found that pancreatic *reg I* gene expression increases in animal models of islet proliferation,[10,15] and decreases in a mouse model of aging.[16] In the latter model, the decrease in *reg I* expression paralleled decreases in insulin and glucose transporter-2 mRNA, both considered critical for normal β-cell function. These studies suggest that *reg I* expression increases during β-cell proliferation, and also may be critical for the general maintenance of islet integrity. This theory has been strengthened by our recent finding that human *reg I* protein is mitogenic to the ARIP ductal cell line and the β-cell line RIN 1046–38.[5] We have also found human *reg I* to be mitogenic to primary cultures of rat pancreatic ductal epithelium in culture, but much less so than on the ductal cell line.[17] Because *reg I* is predominantly a product of the acinar pancreas, we hypothesize that it may act as a paracrine or humoral growth factor on either pluripotent ductal epithelial cells or β-cells themselves. These theories are controversial; there are investigators who believe that *reg I* is not involved in islet growth and development, and is simply a protein expressed after pancreatic manipulation.[18]

Factors that control production of this acinar cell product are unknown. In addition to being induced during pancreatic regeneration following resection or pancreatitis, *reg I* mRNA has been found to be ectopically expressed in areas of the gastrointestinal tract during times of dedifferentiation, specifically in colon and rectal tumors.[4] It is not normally found in colonic or rectal mucosa. Some authors have therefore proposed that *reg I* gene expression may be involved in gastrointestinal tumorigenesis,[4] and maybe a marker of differentiation of gastrointestinal epithelia.[3]

We postulated that *reg I* mRNA and protein levels might be a potential marker of the state of pancreatic cell differentiation. We used the cell line AR42J as a model to correlate acinar cell differentiation with *reg I* gene and protein expression. AR42J is a well established *in vitro* model of acinar cell differentiation; treatment with 10 nmol/L dexamethasone causes them to differentiate: both morphometrically and biochemically.[6] This characteristic has been used to study various molecular changes in acinar cell differentiation.[7,8,19]

After treatment of AR42J cells with dexamethasone, we noted specific morphologic changes, that is, cells flattened and vacuoles appeared. In addition, thymidine incorporation decreased, and *elastase I* gene expression increased. These findings are consistent with those of Logsdon and associates who noted, along with these changes, an increased density of endoplasmic reticulum and secretory granules.[6] We then studied expression of *reg I* mRNA in AR42J cells after induction of differentiation by exposure to dexamethasone. Ten nanomolars of dexamethasone resulted in a statistically significant decrease in *reg I* gene expression.

After induction of differentiation by dexamethasone, quantitative analysis of cellular lysate from AR42J by indirect enzyme-linked immunosorbent assay with a polyclonal antibody also showed a significant decrease in cellular *reg I* protein. Interestingly, other studies showed no change in *reg I* levels in the media (data not shown). This is possibly due to the fact that the protein was not efficiently secreted from this cell line.

These data suggest that the acinar cell product *reg I* is expressed in AR42J cells in a manner opposite to the cell's differentiated state. Furthermore, modulation of *reg*

I gene expression is affected by dexamethasone, presumably through a glucocorticoid receptor. A glucocorticoid responsive element has not been documented in the promoter region of the *reg I* gene,[1-3,20] so it is unknown if this is a direct or indirect effect.

To determine if *reg I* expression could be modulated through gastrointestinal hormonal cell surface receptors, we then measured *reg I* mRNA levels after exposing cells to 10 nmol/L of gastrin and cholecystokinin. AR42J cells express both gastrin (CCK-B) and cholecystokinin (CCK-A) receptors.[21-24] As evidenced by increased thymidine incorporation, gastrin but not CCK was mitogenic to AR42J cells. Others have observed similar results on this cell line.[21] Neither hormone, however, had any significant effect on *reg I* gene expression. Similarly, treatment of the cells with glucagon did not affect *reg I* gene expression. These data indicate that we could not modulate the *reg I* via the gastrin or CCK surface-based receptors, nor through other gastrointestinal hormones, which typically affect acinar proliferation or secretion. We were able to modulate gene expression only through the glucocorticoid receptor.

We conclude that *reg I* is potentially a specific cellular marker of the state of pancreatic cell differentiation, but not of cellular proliferation. This, combined with the fact that *reg I* mRNA can be found ectopically in the gastrointestinal tract during dedifferentiation,[4] suggests that it may be a marker of other gastrointestinal epithelial cell differentiation.

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